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POSSIBLE GROUPS OF AUTOMORPHISMS

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The identity and the group of order 2 are obviously the only groups whose group of automorphisms is the identity since every non-abelian group has a non-cyclic group for its group of inner automorphisms and every abelian group whose order exceeds 2 admits outer proper automorphisms. If a group has the group of order 2 for its group of automorphisms it must be abelian and hence its automorphism of order 2 must result from the automorphism in which every operator corresponds to its inverse since the group of automorphisms of every non-cyclic abelian group is known to be non-abelian.[1] Hence there are three and only three groups which have the group of order 2 for their group of automorphisms, viz., the group of order 3, and the cyclic groups of orders 4 and 6, respectively. The last of these groups is the direct product of two groups and illustrates the elementary theorem that a necessary and sufficient condition that the direct product of two proper groups (groups which differ from the identity) has the same group of automorphisms as one of these groups is that this one involves no subgroup of index 2 and that the other is of order 2.

We proceed to prove that there is no group which has a cyclic group of odd prime power order as its group of automorphisms.[2] The group of order 3 is obviously the group of lowest order which comes under this theorem and it is the group of lowest order which is not the group of automorphisms of some group. It is well known that the group of automorphisms of the group of prime order p is the cyclic group of order $p - 1$ and this could not be a cyclic group of odd prime power order but it may be a cyclic group whose order is a power of 2. It was noted above that the group of automorphisms of a non-cyclic abelian group is non-abelian and hence such a group could also not have for its group of automorphisms a cyclic group of odd prime power order. Finally, a non-abelian group could not have a cyclic group as its group of automorphisms since its group of inner iso-

morphisms is not cyclic and every subgroup of a cyclic group is itself cyclic. This completes a proof of the theorem that *no group has for its group of automorphisms a cyclic group of odd prime power order and that the only groups which have cyclic groups of order 2^m as their group of automorphisms are those of order $2^m + 1$ whenever this is a prime number.*

If the group of inner automorphisms of a given group G does not coincide with the group of automorphisms of G then the former is an invariant subgroup of the latter. From this well-known theorem it results directly that when a simple group of composite order is the group of automorphisms of G then it is either also the group of inner automorphisms of G and hence it is isomorphic with G with rsepect to the central of G or G is abelian. In the latter case all of the operators of G besides the identity are of order 2 since the automorphism of an abelian group in which every operator corresponds to its inverse is an invariant operator of order 2 in its group of automorphisms whenever the abelian group contains operators whose order exceeds 2. When it involves no such operators this automorphism is the identity. It therefore results that whenever the group of automorphisms of an abelian group is a simple group of composite order then this abelian group is of order 2^m and of type 1^m, and it is known that every such group has a simple group of composite order for its group of automorphisms whenever $m > 2$.

Suppose that the group of automorphisms of G is a prime power group and that G involves Sylow subgroups whose orders are powers of at least two distinct prime numbers. At least one of these prime numbers cannot divide the order of the group of automorphisms of G and the operators of G whose orders are powers of this prime number must appear in the central of G since otherwise the group of automorphisms of G would not have an order which is a power of a single prime number. Hence it results that when the group of automorphisms of G is a prime power group then all the Sylow subgroups of G except possibly one appear in the central of G and if one of these Sylow subgroups does not appear in the central of G its order is divisible by the prime number which divides the order of the group of automorphisms of G. In all cases G is the direct product of its Sylow subgroups and all the Sylow subgroups of G which appear in its central are of prime order. Hence there results the following theorem: *When the group of automorphisms of a given group G is a prime power group then G is the direct product of its Sylow subgroups and all of these Sylow subgroups except possibly one are of prime order. If G involves a Sylow subgroup which is not of prime order then the order of its group of automorphisms divides the order of the group of automorphisms of G.*

From the theorem just noted it results that when the order of the central of such a G is divisible by an odd prime number then this number must be of the form $2^m + 1$ and the order of the group of automorphisms of G must

be a power of 2 since the group of automorphisms of an abelian group is the direct product of the groups of automorphisms of its Sylow subgroups. The number of such odd prime numbers which may be the orders of groups appearing in the central of G has evidently no upper limit. When such a G contains a Sylow subgroup whose order is divisible by the square of an odd prime number then this subgroup must be non-abelian and the order of G is not divisible by a power of any other prime number except that it may possibly be divisible by 2, but when G contains a Sylow subgroup whose order is a power of 2 then this subgroup need not be non-abelian. As an instance of such a group we may cite the direct product of a cyclic group whose order is a power of 2 and a group of prime orders of the form $2^m + 1$.

One of the most interesting facts relating to possible groups of automorphisms is that the symmetric group of degree n is its own group of automorphisms except when n is either 2 or 6. In the former case the group of automorphisms is the identity, as was noted above, while in the latter case the group of automorphisms has an order which is twice the order of the corresponding symmetric group. It may be of some interest to prove here that whenever the degree of the symmetric group is different from 2 and 6 then the group of automorphisms of the direct product of the symmetric group and the group of order 2 is also its own group of automorphisms. To prove this fact it may first be noted that such a direct product has obviously the corresponding symmetric group as a subgroup of its group of automorphisms. As this group contains one and only one subgroup of index 2 it is possible to obtain an automorphism of the given direct product by letting the operators of the alternating group contained in this symmetric group correspond to themselves while the remaining operators correspond to themselves multiplied by the invariant operator of order 2 in the given direct product.

The automorphism obtained in this way is clearly an invariant automorphism of order 2 under the group of automorphisms. As this group contains two invariant subgroups which have only the identity in common it is the direct product of these two subgroups. A necessary and sufficient condition that the group of inner automorphisms of a group is also its group of automorphisms is that the group admits no outer isomorphisms. Hence the statement on page 152 of the *Survey of Modern Algebra* by Birkhoff and MacLane (1941) that the group of symmetries of the square admits no outer automorphisms implies that the abelian group of inner automorphisms of the octic group is its group of automorphisms. If this were true we would have here a very simple answer to the question on page 233 of Hilton's *Introduction to the Theory of Groups of Finite Order* (1908) which is as follows: Can a non-abelian group have an abelian group of automorphisms? This question was answered in the affirmative by the present writer on page 124 of volume 43 (1914) of the *Messenger of Mathematics*.

It may be added that what is now commonly called the group of automorphisms used to be called group of isomorphisms which is a somewhat shorter but less suggestive term. On the contrary the recently proposed term *byvalue* in place of the much older and more widely used term coefficient seems to be more justifiable because it is both shorter and more suggestive than the term which it aims to replace. See W. Krull *Elementare Algebra*, page 5 (1939). It is evident that automorphisms are very closely related to conjugate operations. The outer automorphisms correspond to conjugate elements under operators which transform a group into itself but do not themselves appear in the group. The concept of conjugate elements is very fundamental in the theory of groups. In fact A. L. Cauchy and some of his followers used the term *system of conjugate substitutions* instead of the term group.

[1] Miller, G. A., *Transactions of the American Mathematical Society*, Vol. 1, p. 395 (1900).

[2] On page 38, Vol. 10 (1942) of the *Indian Mathematics Student* it is stated incorrectly that "every group can be considered as a group of automorphisms."

FIXED ELEMENTS AND PERIODIC TYPES FOR HOMEOMORPHISMS ON S. L. C. CONTINUA

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We denote by S a semi-locally-connected continuum;[1] the only type of space used. On S only onto-homeomorphisms $T(S) = S$ are considered. This note lends support to the thesis that, since the cyclic element theory[2] tends to view S as a one-dimensional structure, an orderly approach to the behavior of these onto-homeomorphisms on S can be made through this theory. And that, furthermore, convergence properties[3] together with fixed element conditions[4] may be brought to bear with advantage before the many periodic types for T are distinguished. Reserving the details for publication elsewhere we summarize here a fixed element theorem and some characterizations intended to support these views.

If S is a dendrite (tree) and x is an end-point of S fixed under T then let $p \neq x$ be an end-point and denote the unique arc from x to p by $A(x, p)$. Now $T(p)$ is an end-point and there is a point $q \neq x$ such that $A(x, q) = A(x, p) \cdot A(x, T(p))$. If $T(q) = q$ fails to hold the proper subset relation $A(x, q) \subset T(A(x, q))$ arises from one position for $T(q)$. We ignore the other possibility; it is met by the same argument using the inverse of T. Now

the sum $A(x, q) + T(A(x, q)) + \ldots$ of nested arcs has an arc A as its closure and consequently A has two distinct end-points each fixed under T. These methods suitably altered yield the following theorem stated for $T(S) = S$ but with applications in $T(S) \subset S$.

THEOREM (A). *If $N \neq S$ is an invariant node then there exists in S another invariant cyclic element $E \neq N$.*

If B is invariant under T^k, that is, $T^k(B) = B$, it is sometimes said for brevity that the set B is *T^k-invariant*. If k is the least positive integer giving this equality then the sum of the distinct images of B under iteration of T is the *orbit of B* and k is the *period of B*. In the next theorem the oribit of a cut point indicates something of the behavior of T in general. If p is a cut point of S which is not fixed under T then some component R of $S - p$ contains a cyclic element fixed[4] under T. Hence if $H = \overline{R}$ and $K = S - R$ then H and K are continua such that $S = H + K$, $H \cdot K = p$, and $T(H) \cdot H \neq 0$.

THEOREM (B). *If $T^k(K) \cdot K \neq 0$ and k is the least positive integer giving this intersection then either $T^k(p) = p$, or the T^k-orbit P of p is infinite and the least (T^k-invariant) A-set A which contains P is a cyclic chain having two fixed points as end-points.*

Certain precise consequences can also be stated when this theorem fails to apply. The results so far combine to throw new light on certain properties introduced by Ayres and Whyburn;[5] among other things these properties are now freed from pointwise almost periodicity requirements.

($a1$) If C_1 and C_2 are invariant cyclic elements then every cyclic element in the cyclic chain $C(C_1, C_2)$ is invariant.

($a2$) The sum of all invariant cyclic elements of S is a non-empty A-set.

($a3$) For every pair of distinct cyclic elements E_1 and E_2 such that $T(E_1) = E_2$, the cyclic chain $C(E_1, E_2)$ contains one and only one invariant cyclic element.

($w1$) If X is an invariant A-set in S and $N \neq X$ is an invariant node of X, then there exists a fixed cut point of X.

($w2$) Let H and K be any two continua of S such that $S = H + K$ and $p = H \cdot K$ is a cut point of S. If H and K each meets its image under T then $T(p) = p$.

FIRST CHARACTERIZATION THEOREM. *If $T(S) = S$ has one of these five properties it has all of them.*

By assuming that these properties hold for both $T(S) = S$ and $T^2(S) = S$, that is, in general for all powers from T to T^k, the action of T becomes more clearly defined. We give next a view of this type of situation. If L is the set of all end-points in S and each cyclic element of S in $S - L$ has a finite period then T is said to be *elementwise periodic* on $S - L$. This term is due to Ayres; we introduce a kind of dual concept. If A is an invariant true A-set and each component R of $S - A$ has a finite period

then T is said to be *componentwise periodic* at A; *componentwise periodic* on S provided this local property holds for every A. We say an A-set A is *bordered* or *unbordered* according as it is possible or not possible to select a point p in A and a true cyclic element E such that $p = A \cdot E$. The interior of a set X is written *Int* X.

SECOND CHARACTERIZATION THEOREM. *In order that* $T(S) = S$ *be elementwise periodic on* $S - L$ *it is necessary and sufficient that one of the following conditions be satisfied:*

(*a*) *If* M *is any set in* $S - L$ *having the property that* M *contains each cyclic element of* S *which intersects* M *then* $T(M) \subset M$ *implies*[6] $T(M) = M$.

(*b*) T *is componentwise periodic, and for every positive integer* n, T^n *has one of the five properties of Ayres and Whyburn.*

(*c*) T *is componentwise periodic, and each unbordered* T^n*-invariant* A*-set* A *in* S *admits a contracting*[7] T^n*-approximation which preserves interiority at* A*—that is, for every* A*-set* C *with the property* $A \subset Int\ C$ *there is a third* T^n*-invariant* A*-set* B *such that* $A \subset Int\ B \subset C$.

Elementwise periodicity on all of S, or on the complement of the set of all nodes of S, may be characterized directly from the conditions above. The contracting T^n-approximations are directed toward proving the continuity of orbit decompositions; a type which gives rise to interior (open) transformations. Certain methods for establishing the continuity of decompositions by means of these approximations are illustrated using for brevity a strongly restricted space. This gives the essentials of a proof for the theorem[8] which states that *a period function defined for the points of* S *relative to* $T(S) = S$ *and bounded on each cyclic element yields a continuous orbit decomposition.* However, the presence of disjoint closed invariant sets rather than point-orbits in the illustration points the way to generalizations in this direction.

In the absence of any statement to the contrary page numbers refer to the Colloquium Publication, *Analytic Topology*, by G. T. Whyburn, New York, 1942. See also the references in this book.

[1] P. 19.

[2] Pp. 64–98 for terminology and results used freely below.

[3] P. 69.

[4] P. 242. Theorem of Ayres. *Fund. Math.*, **16**, 332 (1932).

[5] Pp. 247–249.

[6] Compare Theorem (1.2), p. 240.

[7] For an "expanding" approximation, see Theorem (4.7), p. 249. Omitting the p. a. p. assumption this too is a characterization.

[8] Abstract in *Bull. Am. Math. Soc.*, **45**, 82 (1939). Also see Theorem (5.1), p. 251 and Theorem (6.42), p. 258.

ATROPINESTERASE, A GENETICALLY DETERMINED ENZYME IN THE RABBIT

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Introduction.—Hereditary differences in enzyme activity are well known among both plants and animals, particularly in so far as they have to do with irregularities in pigment formation.[10, 13] In animals such differences are not restricted to pigment formation but have to do with many other physiological activities such as the xanthophyllase activity which determines the presence or absence of yellow color of body fat in the rabbit,[1, 11] the amylase activity in the digestive juices of silkworm larvae[7] and the uricase activity which determines the amount of nitrogen excreted as uric acid in the dog.[5] In man alkaptonuria, steatorrhea, hematoporphyria, pentosuria and cystinuria are other biochemical differences which are suspected to have a similar background.

Recently interest has been revived in the observation, first reported by Fleischmann[3] and subsequently confirmed by others in various parts of the world, that the blood of certain rabbits can destroy atropine while that of others cannot. Studies of some properties of the enzyme in the serums of those rabbits capable of hydrolyzing atropine have been carried out by Glick,[4] and Glick and Glaubach[5] have investigated the distribution of the atropinesterase among certain tissues in these animals. The possibility that atropinesterase in rabbit serum is an inherited factor was recognized by Levy and Michel[7] but no supporting data have been forthcoming. The present coöperative investigation is an attempt to determine the genetic properties of the enzyme.

Methods.—Blood samples of sufficient volume to provide 0.5 cc. of serum have been obtained from animals at three months of age or older of the proper matings in the laboratory at Brown, centrifuged to remove the cells and placed in the mail for examination within three days at Newark. The enzyme activity was determined by the manometric method employing the Warburg apparatus in the manner previously described.[4, 5] The activity was expressed as atropinesterase units per 100 mg. of serum. The unit is defined as the amount of enzyme required to liberate 1 c. mm. CO_2 in 300 minutes at 30° in a total volume of 4 ml. in the bicarbonate-Ringer medium containing a concentration of substrate (0.25% atropine sulphate) sufficient to achieve the maximum rate of hydrolysis.

Data and Discussion.—Preliminary examination of five unrelated families showed that about 55 per cent of the 181 rabbits examined possessed the

atropinesterase. This is a considerably higher proportion than previously obtained by other investigators and suggests the existence of racial differences, a fact that becomes more apparent when this population is separated into its constituent parts. Two of the families—III, a New Zealand White race which has been closely bred for more than 10 generations, and the *A* race of Castle, a small-sized multiple-recessive strain—appear to lack the enzyme entirely. The other three families possess it in a high proportion of the individuals. Family V, a pure chinchilla race, also closely bred for 12 generations, and family X, a more heterogeneous race originated by hybridization of the small race with other genetic stocks, produce both enzyme-possessing and non-enzyme-possessing offspring. In the first family, among 50 individuals tested the proportion was equal; in the second, 75 per cent of 87 animals examined were atropinesterase-producing. Only 14 individuals have been examined in family II*c*, of which three did not possess the enzyme. Of these families, III, V and X at least, show significant genetic differences. No entire family was found which does not produce some individuals lacking the enzyme.

The evidence as to the hereditary nature of these differences is as follows. In a total of 69 offspring obtained from parents lacking the enzyme, none have shown any trace of the enzyme. Parents possessing the enzyme, on the other hand, may or may not transmit the character to their offspring. If those which do possess it are paired with mates which do not, one of two alternatives follows. Either the offspring *all* possess the enzyme or they are about equally divided between those which possess it and those which do not. This indicates homozygosity in the first case and heterozygosity in the second case, of the parent possessing the character. Thirty-seven individuals have been obtained from the first type of mating, all of which were positive. In a population of 173 individuals obtained from the second type of mating, 88 possessed the enzyme in their blood and 85 did not, which is a close approximation to the equality expected from a monohybrid backcross. From known hybrids mated with each other, 68 young possessed the enzyme and 30 lacked it. This departure from the expected 3:1 ratio is not significant since it is less than twice the probable error.

From these results it appears that the ability to produce the enzyme is dominant over its absence. Dominance is probably not complete, however, since animals known to be heterozygous show a lower mean value of enzyme production than those which are homozygous. For 25 animals known to be heterozygous the mean value was 107 with a range of 52–174. For 4 animals known to be homozygous the mean value was 271 with a range of 232–348.

It is also interesting to note that the enzyme is not present at birth but first manifests itself at one to two months of age. Forty-five animals have been bled from the heart at birth and at monthly intervals two or three

times thereafter. Thirty-nine of these possessed at least one homozygous parent and hence could be expected to have the enzyme in their blood at three months of age. Six were from a heterozygous male and a non-enzyme mother. None of those examined at birth had demonstrable activity in their blood. Twelve out of 16 of the individuals of the first group, examined at the end of the first month, possessed the enzyme in substantial amounts and two others had a trace. All of them were active at subsequent examinations. Of the second group none possessed the enzyme at the end of the first month but one of them did show it at later examinations.

Similar quantitative differences and also differences in the age of production of the enzyme are apparent in the amylase of the digestive juice of the silkworm,[7] and in several enzymes of the pig.[8]

Through information supplied by Dr. C. I. Wright of the National Institute of Health, Bethesda, Md., who had previously discovered the enzy-

TABLE 1

EVIDENCE FOR GENETIC LINKAGE BETWEEN GENES *As* AND *E*

Backcross progeny from F^1 double heterozygote × double recessive

	NON-CROSSOVERS		CROSSOVERS		POPULATION
	E As	*e as*	*E as*	*e As*	
Coupling	10	12	4	4	30
	E as	*e As*	*E As*	*e as*	
Repulsion	4	5	1	2	12
Totals	31		11		42

The deviation from equality, 10, is 4.5 times the P.E., 2.19, and so clearly significant. The indicated crossover percentage is 26.2 ± 5.2.

matic hydrolysis of certain morphine derivatives,[12] there is reason to believe that the enzyme which hydrolyzes atropine may be identical with that which hydrolyzes monoacetylmorphine. Nineteen serums from our laboratory and 8 from his own have been examined by Dr. Wright. Each sample was examined for the hydrolysis of both substrates under the same conditions. All of the 17 individual bloods which hydrolyzed the one compound also hydrolyzed the other, whereas those of 10 individuals hydrolyzed neither. According to Dr. Wright, the order of activity is the same when the serums are arranged as to enzyme concentration for either "monoacetylmorphinease" or "atropinesterase." The initial rate of hydrolysis of the former is somewhat greater than for the latter but the time required for complete hydrolysis is approximately the same, due to the difference in the order of the two reactions.

For the gene responsible for atropinesterase production we shall use the symbol *As* and for its recessive allele, *as*. This gene apparently is borne

on the same chromosome of the rabbit as is the gene *E* for the extension of black pigment in the coat, for in matings between a double recessive individual and an F_1 double heterozygote, crossover recombinations are significantly fewer than non-crossovers, the two classes being 11 and 31, respectively (table 1), whereas equality would be expected if no linkage existed. Tentatively the genes *As* and *E* are regarded as members of a sixth linkage group of the rabbit. Segregation of both of these pairs of genes in this population is entirely regular when they are considered separately.

We regret that due to the national emergency, which has made it impossible for one of us to continue the work, a more accurate determination of the actual strength of this linkage association cannot be obtained at this time. In comparison, however, similar data have been obtained from crosses involving combinations of *As* and the agouti gene *A* and the color gene *C*. Nine and 8 offspring, respectively, have been obtained from these matings and the offspring are as equally divided between crossover and non-crossover combinations as possible.

Considering the population as a whole there is an obvious tendency for females to manifest greater enzyme activity than males, although no significant difference in the distribution of the character to the two sexes is manifest. Close scrutiny of the individual matings, however, reveals that in the backcross matings (*Asas* × *asas*) this tendency is quite pronounced, the average male and female titers being 98 and 129, respectively, and there is a statistically significant tendency for females to possess the character more often than males. A similar situation occurs in the F_2 although less pronounced. The full meaning of this observation is not apparent. It seems probable, however, that possession of atropinesterase is not in itself a sex-linked character since in the same backcross population sufficient numbers have been obtained to indicate that it makes no difference whether the *As* gene is derived from the mother or the father. It seems more probable that this peculiarity is the result of a difference in the genetic milieu of the two sexes, perhaps acting secondarily through the medium of the sex hormones. It is interesting to compare these results with the similar behavior of cholinesterase in rats and mice in which age and also sex are important factors.[2]

Conclusion.—Rabbits which have in their blood serum an enzyme capable of hydrolyzing atropine (and monoacetylmorphine) inherit that peculiarity in a gene (*As*) borne in the same chromosome as the gene (*E*) for the extension of black pigment in the coat. The gene (*As*) is incompletely dominant, homozygotes producing the enzyme more effectively than heterozygotes. The enzyme is not present at birth but appears first at about one month of age, and tends to occur in greater concentration in females and to be demonstrable in a higher percentage of them than in males.

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NOTE ON THE TIME-INTENSITY FACTOR IN RADIOBIOLOGY

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In many radiobiological reactions the effect of a given dosage of radiation is found to depend on the "time-intensity factor," that is, to be a direct function of the intensity ("intensity effect") and to be lower when the treatment is intermittent than when it is continuous, the intensity remaining the same ("fractionation effect"). This phenomenon has been generally attributed to recovery of the biological material from the action of the radiation. The intensity effects have been determined by comparing the results of continuous irradiation with constant dose and different intensities. They have been the object of most experimental and theoretical investigations in this field, although they do not seem to supply, *per se*, crucial information on the course of recovery. In a recent study of the intensity effect on x-ray induced chromosomal aberrations in Tradescantia microspores,[1] one of us (L. D. M.) came to the conclusion, to be amplified and generalized in this note, that fractionation experiments, consisting of two high-intensity irradiations separated by a variable intermission, should be better suited to the investigation of the time-intensity factor. This is because in fractionation experiments radiation can usually be delivered in a short time, during which recovery is negligible, and recovery

may be allowed to take place mainly during the intermission. The application of more than two separate irradiations seems to constitute an unnecessary complication. The basis for this conclusion was an analysis of the assumed "two-hit" mechanism of production of chromosomal rearrangements.

It has been postulated by Sax[2] that chromosomal breaks are produced by x-rays in Tradescantia microspores at a rate proportional to the dosage, and that an observable rearrangement occurs when two or more breaks are available at the same time in the same microspore. The dependence on the time-intensity factor has been attributed to recovery (or "restitution") of single breaks which otherwise would have contributed to the formation of rearrangements. Since previous experiments had not succeeded in yielding definite information on the process of restitution, the following experimental design was suggested as the most efficient. Microspores should be subjected to an x-ray dose D_1, followed by an intermission of duration t and then by a second dose D_2, each dose being delivered within a short time, during which restitution of single breaks is negligible.[1] In the same study a mathematical formula was derived that permits one to gather substantial information from data accessible through an experiment of this type. The derivation of this formula was based, however, on very special assumptions, and the wider scope of its application was not immediately realized. It is the purpose of this note to derive first an analogous formula starting from less special assumptions, and then to proceed to discuss how the same formula, as well as related ones, can be applied to a broader class of phenomena.

I. The fraction of microspores, $f(D_1)$, in which an observable rearrangement is induced by a single dosage D_1, delivered within a sufficiently short time, is a function of the dosage only, and can be determined experimentally. The fraction of microspores in which an observable rearrangement is induced by a fractionated treatment such as that described above is a function of both dosages and of the intermission, $F(D_1, t, D_2)$, that can also be determined experimentally. We shall discuss the dependence of F on the various factors involved, on the basis of the previously postulated mechanism.

After the first dose there will be a fraction $f(D_1)$ of the microspores in which two or more breaks have been produced within a short time so that a rearrangement will certainly result. There will also be another fraction, which we indicate by $g(D_1)$, wherein only one break has been produced. During the intermission, some of the single breaks will undergo restitution. By the time the second dose is given, only a fraction of the initial number of single breaks is left; if this fraction depends on the duration t of the intermission we may indicate it by $r(t)$. Experimental determination of this fraction $r(t)$ is the actual object of the investigation considered here.

At the start of the second irradiation there will be three groups of microspores: (1) a fraction $f(D_1)$ which will show rearrangements; (2) a fraction $g(D_1)r(t)$ in which one single break is still present, and (3) the remaining fraction $1 - f(D_1) - g(D_1)r(t)$ in which no break exists. The effect of the second dose D_2 on this complex population can then be computed as follows. No effect of the first dose is present in group (3), so that a completely new rearrangement requiring two or more breaks will be produced in a fraction $f(D_2)$ of it. In group (2) it is sufficient to produce one or more new breaks to have a rearrangement: The second dose (D_2) will produce in this group one break in a fraction $g(D_2)$ and two or more breaks in a fraction $f(D_2)$. The total fraction of rearrangements out of group (2) is, therefore, $g(D_2) + f(D_2)$. The entire group (1) will be scored anyway as showing a rearrangement, so that it will not show any apparent effect from the second dose. The final scoring of rearrangements will thus include a fraction $f(D_1)$ of all microspores, contributed by group (1), a fraction $g(D_1)r(t)[g(D_2) + f(D_2)]$ contributed by group (2), a fraction $[1-f(D_1)-g(D_1)r(t)]\,f(D_2)$ contributed by group (3). Therefore the total effect of the fractional treatment on the whole microspore population can be written as:

$$F(D_1, t, D_2) = f(D_1) + g(D_1)r(t)[g(D_2) + f(D_2)] + [1 - f(D_1) - g(D_1)r(t)]\,f(D_2)$$

$$= f(D_1) + f(D_2) - f(D_1)f(D_2) + g(D_1)r(t)g(D_2) \qquad (1)$$

It is seen now that this quantity $F(D_1, t, D_2)$ consists of two parts. One part, that we call

$$\varphi(D_1, D_2) = f(D_1) + f(D_2) - f(D_1)f(D_2) \qquad (2)$$

depends only on the dosages D_1 and D_2 and on the experimentally determinable function f. The other part $g(D_1)r(t)g(D_2)$ is proportional to the unknown function $r(t)$ through the unknown quantities $g(D_1)$, $g(D_2)$. On account of the definition of $r(t)$, $r(0) = 1$; the function $r(t)$ can now be calculated in terms of *experimentally determinable quantities:*

$$r(t) = \frac{r(t)}{r(0)} = \frac{g(D_1)r(t)g(D_2)}{g(D_1)r(0)g(D_2)} = \frac{F(D_1, t, D_2) - \varphi(D_1, D_2)}{F(D_1, 0, D_2) - \varphi(D_1, D_2)} \qquad (3)$$

The analogous formula previously derived[1] was based on Swann-Del Rosario's assumption[3] that $r(t) = e^{-\lambda t}$. This new formula does not make use of any such assumption, yet it serves to test directly not only whether $r(t) = e^{-\lambda t}$ but also whether $r(t) = 1$ for $t < \tau$, $r(t) = 0$ for $t > \tau$ (τ being the lifetime of a chromosome break), as suggested by Lea[4], or is a still different function.

II. In the case of complete restitution of single breaks which may, but

need not, occur when t is very large, we should have $r(t_\infty) = 0$, and hence $F(D_1, t_\infty, D_2) = \varphi(D_1, D_2)$. This indicates that the quantity $\varphi(D_1, D_2)$ defined by formula (2) is the total effect of a fractionated treatment under the specific condition that at the start of the second dose all microspores carry either a rearrangement or no break at all.

Whenever the total effect F of a fractionation experiment exceeds φ, it is because at the start of the second dose some microspores carry one single break, that is, show an intermediate, not directly observable, effect of the first dose. This effect, however, becomes experimentally detectable as a sort of "sensitization," because the effect of the second dose on these broken microspores is greater than it would be on microspores carrying no break. The difference between F and φ may then be taken as a new function S measuring the effect of sensitization:

$$S(D_1, t, D_2) = F(D_1, t, D_2) - \varphi(D_1, D_2) = F(D_1, t, D_2) - f(D_1) - f(D_2) + f(D_1)f(D_2) \quad (4)$$

(the latter equality derives from (2)). Comparing (4) with (1) it follows that:

$$S(D_1, t, D_2) = g(D_1)r(t)g(D_2). \quad (4')$$

Furthermore, comparing (4) or (4') with (3), it follows that (3) can be written as:

$$r(t) = \frac{S(D_1, t, D_2)}{S(D_1, 0, D_2)} \quad (3')$$

showing that the recovery function $r(t)$ measures the decline of the sensitization effect S.

It is recovery from this partial, not directly observable, effect of sensitization that we are dealing with, and not recovery from the final observable effect. The latter may be either non-existent (according to the nature of the biological material or the criterion chosen as effect) or may be studied and evaluated directly.[5]

III. It is possible to extend this discussion to a broader class of radiobiologic phenomena, whose mechanism need not be similar to the one postulated for Tradescantia microspores. In fact the definition and the experimental determination of the observable effects indicated by $F(D_1, t, D_2)$ and $f(D_1)$ or $f(D_2)$ are perfectly general and in no way dependent on the particular mechanism that is postulated. Whenever a biological effect of radiation is measured as the frequency of occurrence of an observable event, in sufficiently stable biological material, it should be possible to determine the effect of sensitization S experimentally, through formula (4). For a graphical representation of the quantity S in the special case of $t = 0$, see figure 1.

The interpretation of this quantity remains the same as given above, with exceptions to be considered later. The decline of the effect of sensitization S, as the intermission t increases, may be taken as an index of recovery. This index is identical with $r(t)$ (formula (3')) when the theory developed above for Tradescantia can be applied. In general, however, it may depend also on D_1 and D_2 because: (1) the course of recovery may depend on the degree of sensitization achieved by the first irradiation with dose D_1; and (2) the nature of the observed results may be such that the effect of sensitization as determined by this method may depend on the magnitude of D_2. Accordingly one may replace $r(t)$ by a general index:

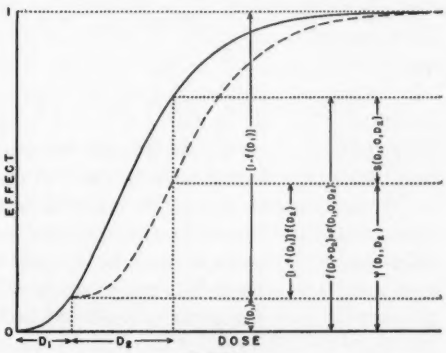

FIGURE 1

Graphical representation of the effect of sensitization as derived from dose-effect relationships.

Dose-effect curve $f(D_1)$ for a single high-intensity irradiation, ————————.

Same curve in a different scale $(1 - f(D_1) : 1)$ referring to a second irradiation, - - - - - - - - -.

When there is an intermission, the total effect $F(D_1, t, D_2)$ falls short of $f(D_1 + D_2)$ and consequently the effect of sensitization $S(D_1, t, D_2)$ falls short of $S(D_1, 0, D_2)$.

$$R = \frac{S(D_1, t, D_2)}{S(D_1, 0, D_2)} \qquad (3'')$$

which can be studied under different conditions.

The experimental determination of the effect of sensitization S for different values of D_1, t and D_2 permits us to reverse the procedure developed for Tradescantia and to test whether or not the mechanism of sensitization and recovery has the same characteristics as the one postulated for Tradescantia. Various analytical or graphical procedures may serve to determine whether or not a function of several variables, such as S in formula (4'), is actually the product of several functions, each of them depending on a single variable. First, the index R may be plotted as a function of t for a number of different values of D_1 and D_2, and it may be verified whether or not all curves so obtained coincide; if they do, R is a function of the interval t only, which may be called again $r(t)$, and S is then the product of its own value at $t = 0$ and of this separate function $r(t)$, namely, $S(D_1, t, D_2) = S(D_1, 0, D_2)r(t)$. The next step is to check whether $S(D_1, 0, D_2)$ itself is the product of two separate functions $g(D_1)$ and $g(D_2)$.

When $t = 0$, the two doses are equivalent to a single dose $D_1 + D_2$; therefore:

$$F(D_1, 0, D_2) = f(D_1 + D_2)$$

and

$$S(D_1, 0, D_2) = f(D_1 + D_2) - f(D_1) - f(D_2) + f(D_1)f(D_2).$$

Thus $S(D_1, 0, D_2)$ depends only on the dose-effect curve for short irradiations and is symmetrical with respect to D_1 and D_2. The quantity $S(D_1, 0, D_2)$ may now be plotted as a function of D_1 for a number of different values of D_2, and it may be checked whether or not all curves so obtained differ only by a change of scale in the ordinates. If they do, $S(D_1, 0, D_2)$ is actually the product of two separate functions $g(D_1)$ and $g(D_2)$, and also the curve representing $g(D_1)$ itself differs from any one of these curves only by a proper choice of the scale in the ordinates (such as to fulfill the equation $S(D_1, 0, D_1) = g^2(D_1)$).

The theory for Tradescantia leading to formula (4′) was derived on the assumption that the production of chromosomal rearrangements takes place according to a "two-hit" mechanism. Conversely, it can be proved mathematically[6] that the function $S(D_1, 0, D_2)$ is a product of $g(D_1)$ and $g(D_2)$ when, and only when, the dose-action curve $f(D_1)$ fulfills conditions slightly more general than those postulated for a "two-hit curve" by Blau and Altenburger.[7]

When the effect of sensitization S cannot be represented by formula (4′), it may be possible to postulate a more complex mechanism of sensitization and recovery and to describe its mathematical consequences by the methods used above for Tradescantia. The effect of sensitization S should then be represented by a sum of terms, each of which has the form indicated in (4′). Experiments aimed at gathering information about the mechanism of sensitization and recovery should therefore determine first the function $S(D_1, t, D_2)$ and next try to break it up into such a sum of terms.

IV. Cases must be considered, in which the function S can still be defined and determined experimentally through formula (4′), but its interpretation as an effect of "sensitization" is not straightforward. For instance, there might be some secondary effect of radiation resulting in partial inhibition of—or resistance to—the main observable effect. This secondary effect, although started by the first dose, could become increasingly intense as subsequent phenomena develop during the intermission, so as to act by itself as a time-intensity factor. In such a case the function S would not measure the "sensitization" proper, but the difference between the sensitization effect and the opposite "inhibition" effect. If the latter effect becomes more important than the former, the function S

becomes negative, which would not be expected to occur on the basis of our earlier considerations.

Another difficulty arises from the possibility that the treated material is initially variable in its sensitivity to radiation. If so, the first dose would induce a final, observable, effect preferentially in the most sensitive organisms, while the second dose would act on a population already selected for higher resistance to radiation. This situation results in an apparent reduction of the effect of sensitization. An effect of this type is important whenever the variability of the material significantly affects the shape of the dose-effect curve for short treatments. This might be the case in experiments like those of MacComb and Quimby,[8] on the production of erythema in human skin: the percentage of patients showing pigmentation plotted against the dosage of a short treatment yields a threshold-type curve, in which, however, the threshold is not ideally sharp. It seems a reasonable interpretation that the threshold would actually be very sharp except for individual variability. In the discussion of the time-intensity factor, one may try to eliminate the effect of variability, by estimating what the experimental results would be in absence of variability. This is frequently done in the case of threshold-type curves, by discussing the ideal case of a perfectly sharp threshold instead of the observed one. When the threshold is ideally sharp, the observable quantities, i.e., $f(D_1)$, $f(D_2)$, $F(D_1, t, D_2)$, can be only zero or unity, and hence the same holds for the derived quantities $\varphi(D_1, D_2)$ and $S(D_1, t, D_2)$. The purpose of experimental investigation should then be to determine all the possible ways of giving a threshold treatment. Each threshold treatment consists of a set of values D_1, t, D_2 lying on the boundary at which the effect of sensitization jumps from zero to one. Such an investigation was actually carried out by MacComb and Quimby, except that these authors kept the two doses equal ($D_1 = D_2$) and thus did not determine the whole range of threshold treatments but only a cross-section of it. For instance, in order to test Lea's theory[9] on the elimination of poisonous substances for the case of skin erythema, D_1 should rather be kept constant, and then it may be determined how large the second dose D_2 must be, as a function of the duration t of the intermission, in order to achieve a threshold reaction.

Summary.—X-ray treatments consisting of two irradiations, delivered within a sufficiently short time and separated by variable time intervals, seem to be most suitable for the investigation of those recovery processes that express themselves through the time-intensity factor. From experimental data thus obtained, an index of recovery R can be calculated, which should prove useful in studying the course of recovery. Experimental tests are suggested for determining the complexity of the process. The discussion leads to a more comprehensive point of view with reference to various radiobiological theories previously proposed.

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SOME INTERRELATIONSHIPS OF PYRIDOXINE, ALANINE AND GLYCINE IN THEIR EFFECT ON CERTAIN LACTIC ACID BACTERIA

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Various investigators[1, 2, 3] have shown that pyridoxine (vitamin B_6) under certain conditions is a necessary growth factor for certain species of lactic acid bacteria. One such organism is *Streptococcus lactis* R. In a detailed study of the requirement of this organism for pyridoxine, Snell, *et al.*,[4] showed that there existed in natural products a substance which could be derived from pyridoxine by metabolic processes, which far surpasses pyridoxine in its physiological activity for this organism. To distinguish this substance from synthetic pyridoxine it was tentatively called "pseudopyridoxine." It was later shown[5] that if heat sterilization were avoided, pyridoxine was almost without growth-promoting activity for this organism. It became highly active on autoclaving with the basal culture medium. The degree of activity increased as the period of heating was increased. The effect was traced to interaction during autoclaving between pyridoxine and the amino acids of the medium; presumably "pseudopyridoxine" or a substance of similarly high activity for the test organism was formed in small amounts.

While investigating the effect of individual amino acids in promoting the above "activation" of pyridoxine during heating, it was found that large amounts of alanine completely replaced pyridoxine as a growth factor for this organism. Glycine, on the other hand, inhibited growth. This inhibitory effect of glycine was removed, within limits, by additional amounts of pyridoxine, but not by additional amounts of any of the other vitamins. It was similarly removed—even more effectively—by alanine,

but not by other amino acids. Details of these findings are presented below.

Experimental.—The basal medium used, and the conditions of testing, were exactly similar to those previously described.[4] The test organism was in all cases *Streptococcus lactis* R. Table 1 shows the comparative effects of pyridoxine, *dl*-alanine and glycine on growth. The maximum growth effect of pyridoxine is reached under these conditions at a concentration of 3 γ per 10 cc. of medium. *dl*-Alanine promotes growth to the same level when 1 to 3 mg. is added per 10 cc. This comparatively low activity of alanine suggests that its activity might be due to contamination with a substance possessing pyridoxine activity. That this is not the case is shown by the following considerations. Synthetic *dl*-alanine obtained from three different sources had exactly the same activity. This activity was unchanged after solution in water, treatment with an equal weight of activated charcoal, evaporation and recrystallization. Finally, the activity of *dl*-alanine was destroyed by nitrous acid treatment; pyridoxine is unaffected by such treatment.[6] Separate experiments showed that *dl*-alanine would support continued heavy growth of the organism through repeated subcultures in the complete absence of pyridoxine. Under the conditions described, therefore, alanine in high concentration completely replaces pyridoxine in its growth effect on this organism.

TABLE 1

THE EFFECT OF PYRIDOXINE, ALANINE AND GLYCINE ON GROWTH OF *Streptococcus lactis* R.

SUBSTANCE TESTED	AMOUNT ADDED γ PER 10 CC.	GALVANOMETER READING*
.....	.....	20
Pyridoxine hydrochloride	0.2	30
	0.5	44
	1.0	55
	3.0	68
	10.0	68
dl-Alanine	100	21
	300	32
	500	53
	1000	68
	3000	73
Glycine	1000	8.8
	5000	2.8

* Distilled water reads zero; a reading of 100 is complete opacity.

Addition of glycine to the basal medium suppressed growth below that which occurred in the control tubes. Slight growth in such unsupplemented tubes occurs because of carry-over with the inoculum of small amounts of

substances which have pyridoxine activity, or the incomplete removal of such substances from the hydrolyzed casein of the basal medium. When extreme care is taken in preparing the basal medium, and small, washed inocula are used, no growth occurs in the unsupplemented medium, and addition of glycine to such a medium results in no apparent change. The inhibitory effect of glycine is more clearly seen when maximum growth of the organism is permitted by the addition of pyridoxine or of alanine. Results of such an experiment are shown in table 2. From 1 γ to 3 γ of pyridoxine hydrochloride was sufficient to permit growth to proceed to a maximum. No greater growth was attained when as much as 300 γ of the vitamin was added. In the presence of only 1 γ of the vitamin addition of 2 mg. of glycine suppressed growth to approximately the level reached in the control tubes. As the amount of pyridoxine is increased, the amount of glycine required to suppress growth to the level of the control tubes increases: thus with 3 γ pyridoxine hydrochloride, 5 mg. of glycine is required; with 10 γ pyridoxine nearly 10 mg. of glycine is required; with 30 γ of pyridoxine, 30 mg. of glycine is required for inhibition. The inhibitory effect of glycine is thus overcome to a very considerable extent by the presence of pyridoxine in large amounts. More than 30 mg. of glycine is apparently irreversibly toxic for this organism under these conditions. Just as alanine replaces pyridoxine in its growth effect for this organism, so it

TABLE 2

INHIBITORY EFFECT OF GLYCINE ON GROWTH OF *Streptococcus lactis* AND ITS REVERSAL BY PYRIDOXINE AND ALANINE

ADDITIONS TO CULTURE MEDIUM			ADDITIONS TO CULTURE MEDIUM		
GLYCINE MG. PER 10 CC.	PYRIDOXINE HYDROCHLORIDE, γ PER 10 CC.	GALVANOMETER READING*	GLYCINE, MG. PER 10 CC.	*dl*-ALANINE, MG. PER 10 CC.	GALVANOMETER READING*
0	0	19	0	0	16
0	1	58	0	1	69
0	3	64	0	3	73
0	10	63	0	10	73
0	30	60	0	30	73
0	300	58	1	1	65
1	1	36	3	1	41
2	1	21	10	1	10
2	3	50	1	10	73
5	3	21	3	10	72
10	3	3	10	10	71
2	10	60	30	10	66
5	10	43	100	10	12
10	10	15	100	30	12
5	30	58	...	..	..
10	30	38	...	..	..
30	30	18	...	..	..
30	300	14	...	..	..

* As in table 1.

also reverses the inhibitory effect of glycine. In this experiment, 1 to 3 mg. of alanine permitted maximum growth to occur. With 1 mg. of *dl*-alanine present, about 10 mg. of glycine was required to reduce growth to a level below that reached in the control tube. With 10 mg. of *dl*-alanine, 100 mg. of glycine was required to produce the same effect. Although pyridoxine on the weight basis is much more active than alanine in effecting reversal of glycine inhibition, alanine reverses this inhibition when the glycine concentration is higher. When 100 mg. of glycine is used, the inhibition is not reversed by additional amounts of alanine.

The specificity of these responses is shown in table 3. Of the water-soluble vitamins tested, only pyridoxine was effective in promoting growth on this medium and reversing growth inhibition produced by glycine. Similarly, each of the amino acids so far obtained from casein hydrolyzates was tested. Only those fairly closely related to alanine are listed in the table. Of these, only alanine was effective in duplicating the growth effect of pyridoxine, and in reversing growth inhibition produced by glycine. Each of the other amino acids was completely ineffective in this respect. Serine and threonine show slight inhibitory effects in the same direction as does glycine.

The effectiveness of various compounds related in structure to glycine in producing inhibition of growth was further tested. These results are given in table 4. β-Alanine, *dl*-serine and *dl*-threonine inhibited growth, but their effectiveness was considerably less than that of glycine. In each case the growth inhibition was prevented by simultaneous addition to the medium of additional amounts of pyridoxine or of alanine. As with glycine (table 2), this inhibition was reversible only within a limited range of concentrations, above which inhibition of growth by these compounds was not reversed by additional amounts of pyridoxine or of alanine.

The fact that added alanine replaces pyridoxine for *S. lactis*, but growth does not occur on the basal medium without pyridoxine or alanine, suggests that this organism is incapable of synthesizing alanine. This seemed rather unusual, since lactic acid is the chief product produced by the organism during growth. The amino acid requirements of the test organism were therefore determined. The hydrolyzed casein of the basal medium was replaced with a mixture of all of the amino acids listed as occurring in casein[7] with the exception of β-hydroxyglutamic acid. The neutralized mixture was added to the medium at such a level that each 10 cc. of medium contained 1 mg. of each amino acid. Ten milligrams of glutamic acid and 3 γ of pyridoxine hydrochloride were added per tube. The effect of omitting individual amino acids from the mixture was then determined. Results are shown in table 5. Alanine, glycine, threonine and serine are among those amino acids which are essential for the growth of this organism. While alanine in high concentration replaces pyridoxine, the addition of excess

TABLE 3

SPECIFICITY OF PYRIDOXINE AND *dl*-ALANINE IN PROMOTING GROWTH AND REVERSING INHIBITION OF GROWTH BY GLYCINE

SUBSTANCE TESTED	AMOUNT ADDED, γ PER 10 CC.	GALVANOMETER READING*	
		UNSUPPLEMENTED	3 γ PYRIDOXINE HYDROCHLORIDE AND 4 MG. GLYCINE ADDED PER 10 CC.
.....	....	10	37
Pyridoxine hydrochloride	3	58	42
Pyridoxine hydrochloride	100	55	55
Thiamin chloride	100	10	37
Riboflavin	100	10	38
Nicotinic acid	100	10	38
Pantothenic acid	100	10	36
Biotin	2	10	38
p-Aminobenzoic acid	100	11	37
Folic acid†	2	10	36
dl-Alanine	1000	58	48
dl-Alanine	5000	60	57
dl-α-Aminoisobutyric acid	5000	10	37
dl-Norvaline	5000	10	37
dl-Valine	5000	9	37
dl-Serine	5000	6	31
dl-Threonine	5000	6	31

* As in table 1.

† Potency 10,000.[23]

TABLE 4

GROWTH INHIBITION BY COMPOUNDS RELATED IN STRUCTURE TO GLYCINE*

COMPOUND TESTED	INHIBITION	AMOUNT REQUIRED,† MG. PER 10 CC.	INHIBITION REVERSED BY	
			PYRIDOXINE HYDROCHLORIDE	*dl*-ALANINE
Glycine	+	2	+	+
β-Alanine	+	30	+	+
dl-Serine	+	15	+	+
dl-Threonine	+	15	+	+
dl-Alanine	−	(100)	..	..
Lactic acid	−	(30)	..	..
Creatine	−	(30)	..	..
Choline chloride	−	(30)	..	..
Benzoyl glycine	−	(30)	..	..
Glycollic acid	−	(30)	..	..

* All compounds were tested for their inhibitory properties by adding increasing amounts to the basal medium to which 0.5 γ of pyridoxine hydrochloride per 10 cc. was added. This amount of pyridoxine is sufficient to produce about one-half maximum growth of *S. lactis* R. under the test conditions.

† The amount of compound necessary to reduce growth to the level in the control tube (without pyridoxine) is given. Where the compound was not inhibitory, the highest level tested is given in parentheses.

pyridoxine does not permit growth in the absence of alanine. Glycine, threonine and serine (table 4) become inhibitory only when added in amounts considerably higher than those which are present in the hydrolyzed casein of the base medium.

TABLE 5

AMINO ACID REQUIREMENT OF *Streptococcus lactis* R.

AMINO ACID OMITTED	GALVANOMETER READING*	AMINO ACID OMITTED	GALVANOMETER READING*
None	51	*d*-Glutamic acid	0
Glycine	18	*dl*-Serine	0
dl-Alanine	0	*dl*-Threonine	0
dl-Alanine†	0	*l*-Arginine	0
dl-Valine	48	*l*-Histidine	49
dl-Leucine	5	*l*-Lysine	2
dl-Isoleucine	50	*l*-Proline	49
l-Phenylalanine	51	*l*-Hydroxyproline	51
l-Tyrosine	42	*dl*-Methionine	49
l-Aspartic acid	10	*l*-Tryptophane	0
		l-Cystine	20

* As in Table 1.

† 3.0 mg. pyridoxine hydrochloride (neutralized) added.

The extent to which these results apply to other lactic acid bacteria has not been extensively investigated. *S. lactis* 125 responds in exactly the same manner to additions of pyridoxine, alanine and glycine as does *S. lactis* R. Pyridoxine is also required for growth of *Lactobacillus casei*[2, 3]; for this organism alanine does not replace pyridoxine. *L. casei* is likewise very resistant to glycine inhibition.

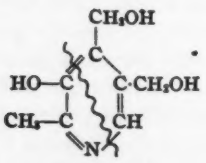
Discussion.—The inhibition of growth of *S. lactis* by glycine, serine, threonine or β-alanine and its removal by alanine is most easily explained as an example of metabolic interference of structurally similar compounds in the sense postulated by Woods[8] and Fildes.[9] Alanine is an essential amino acid, the presence of which is required for growth of this organism; the presence of large amounts of structurally similar amino acids blocks utilization of alanine. Addition of increased amounts of alanine while maintaining the concentration of the inhibiting substance constant again makes alanine available, by simple mass action. The fact that glycine, serine and threonine are themselves likewise essential for growth need not alter this conception; if they interfere with alanine utilization, growth is nevertheless prevented. McIlwain[10] has shown that such interference may exist between amino acids and their sulfonic acid analogs, while existence of such interference between the sulfonamides and *p*-aminobenzoic acid[8] or between pantothenic acid and pantoyl-taurine[11, 12] is on firm experimental grounds. Similar cases of interference between amino acids have

been previously noted. Thus Gladstone,[13] investigating the amino acid nutrition of *Bacillus anthracis*, found that valine or leucine added singly to a mixture of amino acids able to support growth without them completely prevented growth. The "toxic" effect of valine could be counteracted by addition of leucine, and vice versa. Similar interrelationships were found between valine and threonine, valine and α-aminobutyric acid, and threonine and serine.

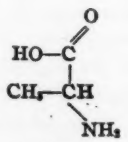
The action of pyridoxine in alleviating toxicity due to glycine is not easily explained on this basis. Apparently, glycine blocks utilization of pyridoxine in some manner; additional amounts of pyridoxine counteract this effect. Indeed, counteraction of glycine inhibition by alanine and pyridoxine may be due to the same mechanism, since these compounds replace one another in their growth effect, presumably by permitting formation of the same physiologically active compound by the test organism. Somewhat analogous cases are known: methionine[14] and the purine bases[15, 16] can under certain circumstances reverse growth inhibitions produced by the sulfonamides, a result produced most effectively by *p*-aminobenzoic acid. In the absence of the sulfonamides, each of the above compounds may promote growth in the same manner.[16]

Various cases in which glycine[17, 18, 19] and serine[20] were toxic when administered to animals have been reported. It would be of considerable interest to see if the toxicity of these compounds could be alleviated in these cases by additional amounts of pyridoxine or of alanine.

The complete replacement of pyridoxine for *S. lactis* by alanine deserves special comment. All experience to date has indicated that the B vitamins occur universally in all forms of living matter.[21, 22] When an organism grows without added supplies, it does so because it is able to synthesize those supplies. According to this view, presence of sufficient quantities of alanine permits the synthesis by *S. lactis* of pyridoxine, or of the active substance derived from pyridoxine.[4, 5] In the absence of such concentrations of alanine, pyridoxine itself or its metabolites are necessary for growth. The mechanism by which alanine replaces pyridoxine is unknown, but it seems quite probable that it may serve as a direct precursor for pyridoxine in the following manner:



Pyridoxine



Alanine

Organisms which are able to utilize alanine in place of pyridoxine would be

able to synthesize the remaining portion of the molecule and couple it with alanine.

Summary.—Alanine in sufficient concentration completely replaces pyridoxine for *Streptococcus lactis*. No other amino acid tested does this. Glycine inhibits growth; threonine, serine and β-alanine are inhibitory at higher levels. Inhibition by each of these substances is counteracted by addition of more pyridoxine to the medium. No other vitamin has this action. Such inhibition is also counteracted by alanine, but by no other amino acid. Possible explanations for these facts are discussed. Alanine may serve as a direct precursor of pyridoxine for *Streptococcus lactis*.

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STRAIN SPECIFICITY AND PRODUCTION OF ANTIBIOTIC SUBSTANCES*

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It has been definitely established[5] that the capacity of various microorganisms to produce antibiotic substances is widely distributed in nature. These substances are largely bacteriostatic and to a less extent bactericidal; some are also fungistatic and fungicidal. The antagonistic properties of an organism are not characteristic of a particular group or even species, but only of certain strains of given species. This was found to hold true, for example, for *Pseudomonas aeruginosa*, the first important antagonistic organism to receive attention. Some strains of this organism are able to produce the antibacterial agent pyocyanase, others form both pyocyanase and pyocyanin, whereas still others either are inactive or have only limited activity. This is also true of the spore-forming bacteria that possess antagonistic properties, only some of the strains being capable of inhibiting the growth of different bacteria. Much[3] even suggested separation of the antagonistic strains of *Bacillus mycoides* into a special group, to be recognized as lysogenic strains. The antibacterial strains of spore-forming bacteria were shown[4] to be characterized by specific physiological properties, such as production of H_2S on peptone media, failure to hydrolyze starch, and gram-negative nature of the cultures when 18 to 24 hours old; the inactive strains did not form H_2S, were able to hydrolyze starch, and were gram-positive in the 24 to 48-hour broth cultures.

The phenomenon of specificity is characteristic not only of bacteria but also of fungi. This is of particular interest in connection with the important organism *Penicillium notatum*, which produces the antibacterial agent penicillin. Some of the strains of this fungus produce penicillin abundantly, whereas others have only limited activity. The strain specificity of this organism applies not only to the quantitative production of penicillin, but also to qualitative differences. Some of the strains produce largely this important substance and very little of a second factor, which is also active against certain gram negative bacteria, such as *Escherichia coli*, and which has, therefore, been designated as the *E. coli* factor,[6] penatin[2] or notatin,[1] whereas other strains show a reverse ratio in the production of these two active substances, especially under certain conditions of culture.[6]

In a survey of the distribution of antagonistic microörganisms in nature, 164 strains of different fungi were isolated from soils, composts and dust.[6]

Two species, *Aspergillus fumigatus* and *A. clavatus*, were selected for further tudy; the first was represented by 16 isolations and the second by 15 strains. Although morphologically the same, the various strains differed greatly in their capacity to produce the specific antibacterial substances fumigacin and clavacin. The differences appeared to be quantitative rather than qualitative in nature; although the amount of the active substance produced on the same medium and under the same conditions varied, the nature of the substance was apparently the same for all strains of the particular organism.

TABLE 1

GROWTH AND PRODUCTION OF CLAVACIN BY *A. clavatus* 129

Shallow Layers (250-Ml. Portions) in 1-Liter Flasks, at 28°C.

AGE OF CULTURE, DAYS	REACTION OF MEDIUM, pH	GLUCOSE CONSUMED, G. PER LITER	NO_3-N IN MEDIUM, MG. PER LITER LEFT	NO_3-N IN MEDIUM, MG. PER LITER CONSUMED	ACTIVITY OF CULTURE, *E. coli* UNITS	CLAVACIN ISOLATED YIELD, G. PER LITER	CLAVACIN ISOLATED ACTIVITY OF MATERIAL,[a] *E. coli* UNITS
0	4.6	..	51.2	0	...	..	.....
3	4.9	1.50	28.4	22.8	30	0.27	120,000
5	5.1	2.82	7.5	43.7	60	0.52	100,000
7	5.0	3.40	4.4	46.8	100	0.63	200,000
10	5.1	3.86[b]	3.0	48.2	100	0.71	200,000
14	5.5	3.86	2.9	48.3	75	0.72	10,000
21	5.9	3.86	..	..	30	0.66	600
26	6.6	3.86	..	..	10	..	.....
36	6.5	3.86	..	..	0	0.62	200

[a] A unit of activity is the ratio of 10 to the amount of material required for 10 ml. of nutrient agar, in order to inhibit completely the growth of the test organisms.

[b] Complete consumption of sugar.

In order to throw further light upon the influence of strain specificity in connection with the production of antibiotic substances, the results of the following experiments with *A. clavatus* are reported here. Of the 15 strains employed in these studies, 3 (Nos. 129, 130, 164) were isolated in this laboratory from composts and 12 were received from Dr. Charles Thom, of Washington, who obtained them from different sources. A simple synthetic medium, usually designated as Czapek-Dox,[6] was used. The course of growth of one of the active strains of this organism, as measured by sugar consumption and nitrogen assimilation, and the rate of production of the antibiotic substance were brought out in table 1. The highest antibacterial activity of the culture corresponded with the maximum growth period, as indicated by the complete utilization of the sugar and maximum consumption of the nitrogen. One may, therefore, conclude that the formation of the antibacterial substance is not a result of autolysis of the fungus mycelium, since after the maximum growth of the fungus was attained, the activity was rapidly reduced. Whether the substance is

produced during the breakdown of the sugar or is synthesized in the cell material and excreted into the medium still remains to be determined.

The active substance, clavacin, was isolated from a definite volume of culture medium at the various periods of growth, by the use of a method described elsewhere.[7] The yield of the material thus isolated reached a

TABLE 2

PRODUCTION OF CLAVACIN BY 15 STRAINS OF *A. clavatus*

NO. OF STRAIN	INCUBATION, DAYS	pH	ACTIVITY OF CULTURE *E. coli* UNITS	ACTIVITY OF CULTURE *B. subtilis* UNITS	YIELD, G. PER LITER	ISOLATED CLAVACIN[a] ACTIVITY, UNITS PER 1 G. *E. coli*	ISOLATED CLAVACIN[a] ACTIVITY, UNITS PER 1 G. *B. subtilis*
120	5	6.5	0	0	0.016	25,000	75,000
120	14	8.4	0	0	...	....	....
121	5	4.2	75	75	1.442	1,000,000	1,200,000
121	13	6.7	100	300	...	....	....
122	5	4.5	0	0	0.035	< 4,000	< 4,000
122	13	8.0	0	0	...	....	....
123	5	4.6	20	20	0.467	120,000	120,000
123	13	4.5	75	100	...	....	....
124	5	6.2	0	0	0.016	< 8,000	< 8,000
124	13	8.4	0	0	...	....	....
125	5	3.2	0	0	0.248	600	10,000
125	13	3.9	0	0	...	....	....
126	5	6.3	0	0	0.039	< 20,000	< 20,000
126	13	8.2	0	0	...	....	....
127	5	7.4	0	0	0.007	< 8,000	< 8,000
127	13	8.1	0	0	...	....	....
128	5	6.7	0	0	0.017	7,000	14,000
128	13	8.0	0	0	...	....	....
129	5	3.6	100	100	0.950	400,000	3,000,000
129	7	4.4	150	250	...	....	....
129	15	6.8	75	100	...	....	....
129T	5	6.6	20	10	0.512	80,000	120,000
129T	15	5.9	75	75	...	....	....
130	5	4.8	< 10	< 10	0.323	500,000	1,400.00
130	13	4.7	20	30	...	....	....
130T	5	6.9	< 10	30	0.050	< 4,000	40,000
130T	15	7.9	< 10	< 10	...	....	....
131	5	6.9	< 10	30	0.035	5,000	25,000
131	15	7.8	< 10	< 10	...	....	....
164	5	4.3	30	25	0.430	1,000,000	2,000,000
164	7	4.6	90	100	...	....	....

[a] Eight-day-old culture used for the extraction of the clavacin.

maximum in 7–10 days, corresponding to the maximum activity of the culture itself. After the maximum was reached the yield of extracted material remained constant, but its activity diminished rapidly, parallel to the reduction in the activity of the culture medium of the organism. This emphasizes the fact that the clavacin produced by the fungus undergoes in the culture some chemical change which results in the destruction of

its activity. The crude, isolated clavacin is also rather unstable, especially under certain conditions as at an alkaline reaction or on complete drying. The nature of the chemical changes thus produced is a matter for further study.

The production of clavacin by the 15 different strains of *Aspergillus clavatus* is illustrated in table 2. These strains differed greatly in their metabolism, as measured, for example, by changes in the pH values of the medium: the reaction became alkaline in some cases, but in others it remained distinctly acid, in the same medium and under the same conditions of culture. There were other marked differences in the growth of the various strains, which need only be mentioned here, such as the intensity of the odor produced, the pigmentation (yellow to orange) of the cultures, and the abundance and rate of appearance of mycelium and of spores. The most significant difference, however, was in the antibacterial activity of the strains, some of which were highly active, whereas others had no activity at all. The three strains that were isolated from stable manure, namely, Nos. 129, 130, 164, produced a highly active substance; the actual yield of this substance differed, however, and the difference was sufficient to account for the variation in the activity of the culture medium. Only three of the cultures obtained from Dr. Thom, namely, Nos. 121, 123 and 129T, gave a high activity and a fair yield of antibacterial substances. The clavacin isolated for the different active strains appeared to be the same, as shown by chemical properties and by its antibacterial spectrum or the specific action against different bacteria. There was only one exception, that of strain No. 130T; whereas all the other strains produced a substance which was 2–5 times as active against *Bacillus subtilis* as against *Escherichia coli*, this strain had little, if any, activity against *E. coli* as compared with a fair activity against *B. subtilis*. On further study, this strain was found to comprise two forms, which may explain the difference in the apparent activity of the antibacterial substance produced.

It is interesting to note that, whereas the reaction of the medium of all the six active strains remained distinctly acid, the reaction of the inactive strains, with the exception of No. 125, changed to alkaline. When large volumes of the culture medium of all the strains were treated alike for the isolation of the clavacin, it was found that even some of the inactive strains yielded some active substance. A liter of medium was treated with Norit A, which was then extracted with ether and chloroform. Some of the inactive strains gave some clavacin, possessing fair activity, as, for example, No. 120; other strains, however, gave a good yield of material with only limited antibacterial activity, as No. 125. Whether this fact proves that even the inactive strains may produce clavacin, which becomes inactivated as a result of unfavorable changes in the medium (reaction, for example), still remains to be determined.

These results warrant the conclusion that a single species of a fungus contains many strains that vary greatly in their capacity to produce antibiotic or antibacterial substances. The variation is both quantitative and qualitative, though the active substance produced by the different strains is apparently the same, chemically and biologically. This tends to emphasize the danger of drawing conclusions regarding the antibacterial activity of an organism based upon type cultures obtained from

TABLE 3

BACTERIOSTATIC ACTIVITY OF THREE CLAVACIN PREPARATIONS

On Basis of 1 G. of Dry Material

TEST ORGANISM	NO. 121	NO. 123	NO. 129
Bacillus subtilis	300,000	250,000	275,000
Bacillus megatherium	150,000	125,000	140,000
Bacillus mycoides	100,000	85,000	100,000
Bacillus cereus	150,000	125,000	100,000
Sarcina lutea	300,000	250,000	275,000
Staphylococcus aureus W2	150,000	125,000	100,000
Staphylococcus aureus D	200,000	165,000	170,000
Staphylococcus aureus H	150,000	83,000	100,000
Staphylococcus aureus W1	150,000	125,000	130,000
Salmonella schottmuelleri	300,000	250,000	200,000
Vibrio cholera-suis	350,000	300,000	270,000
Salmonella sp., Breslau strain	250,000	165,000	265,000
Aerobacter aerogenes	75,000	63,000	80,000
Escherichia coli	175,000	165,000	230,000
Shigella dysenteriae[a]	250,000	...	...
Shigella paradysenteriae[a]	210,000	...	...

[a] Mixed lot of clavacin.

collections. Wilkins and Harris[8] recently reported, for example, on the basis of tests with one culture that *A. clavatus* does not produce any bacteriostatic substance, whereas 40 per cent of all the Aspergilli tested produced such substances. Had other strains of these organisms been employed, totally different results undoubtedly would have been obtained.

It has been shown previously[7] that clavacin is almost as active against gram-negative bacteria as against gram-positive organisms. In order to establish whether this is true for preparations isolated from the different active strains of *A. clavatus*, the results of a typical experiment are presented in table 3. Three clavacin preparations were obtained and found to be alike in their action against a number of gram-positive and gram-negative bacteria, with only minor variations. In all cases, *Aerobacter aerogenes* was less sensitive to the action of clavacin than was *E. coli*, and *B. mycoides* and *B. cereus* were less sensitive than was *B. subtilis*.

Summary.—A study was made of the production of the antibacterial substance clavacin by 15 different strains of the fungus *Aspergillus clavatus*.

The formation of clavacin in the culture medium corresponded with the maximum growth, as measured by the complete consumption of the sugar in the medium and by maximum nitrogen utilization.

After the maximum antibacterial activity had been reached in the medium, the activity of the clavacin was rapidly destroyed. This was found to be due not to the disappearance of the substance itself from the medium but to its inactivation.

Only six of the fifteen strains of *A. clavatus* produced considerable amounts of clavacin. The remaining nine strains gave only traces of the active substance.

The clavacin produced by the different active strains of *A. clavatus* was found to be the same biologically, as shown by its activity against different bacteria, and apparently the same chemically, as shown by solubility studies and chemical behavior.

Attention is directed to too hasty generalizations concerning the ability of certain fungus species to produce antibacterial substances, based upon the study of single strains of a given organism.

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THE DETERMINATION OF L.D.50 AND ITS SAMPLING ERROR IN BIO-ASSAY

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In the bio-assay problem we have two methods in the literature: (1) the probit method which has been actively promoted by C. I. Bliss, though suggested in its essentials by Gaddum, and (2) the 50% end-point method of Reed and Muench, which, again, was developed from earlier sugges-

tions.[1] Both of these methods depend fundamentally on the assumption that the probability of an effect being realized in an infinite population to each of which a dose D has been administered is given by the probability integral

$$P = \frac{1}{\sqrt{2\pi}\sigma}\int_{-\infty}^{x} e^{\frac{-(x-\mu)^2}{2\sigma^2}}dx,\ x = \log D, \qquad (1)$$

where μ = L.D.50 is the dose measured as the natural logarithm of D which affects just one-half of the population, and σ is a measure of the scatter of the effect, a sort of inverse of the homogeneity of the population with respect to the effect; and both methods depend further on an arithmetic-graphic treatment to smooth out statistical fluctuations, due to small numbers in the experimental samples.

To examine a number of matters to which we desire to call attention it will be simpler to replace the probability-integral (1) by the growth curve which between wide limits does not depart seriously therefrom; indeed it is known that the value of P does not precisely follow (1), sometimes departing considerably from it, and it is by no means certain that the growth curve would not fit the observations as well as the probability integral curve. If D be the dose we may then write

$$P = \frac{D^{2\alpha}}{D^{2\alpha} + e^{2\alpha\gamma}} = \frac{1}{1 + e^{2\alpha(\gamma - x)}} = {}^1/_2 + {}^1/_2 \tanh \alpha\,(x - \gamma)$$

where $x = \log D$ and tanh is the hyperbolic tangent. The constant γ = L.D.50; the constant α is a measure of the homogeneity of the reaction of the population. If P_1 and P_2 be the percentages of the population which respond to doses D_1 and $D_2 = aD_1$ so that $x_2 = x_1 + \log a = x_1 + c$ we have

$$\frac{1}{P_1} = 1 + e^{2\alpha(\gamma - x_1)},\ \frac{1}{P_2} = 1 + e^{2\alpha(\gamma - x_1 - c)}$$

$$\log\frac{Q_1}{P_1} = 2\alpha(\gamma - x_1),\ \log\frac{Q_2}{P_2} = 2\alpha(\gamma - x_1 - c)$$

where $Q_i = 1 - P_i$ and

$$\alpha = \frac{1}{2c}\left[\log\frac{Q_1}{P_1} - \log\frac{Q_2}{P_2}\right],\ \gamma = \frac{x_1 + x_2}{2} + \frac{1}{4\alpha}\left[\log\frac{Q_1}{P_1} + \log\frac{Q_2}{P_2}\right]. \qquad (2)$$

If then we take $P_1 = 0.25$ and $P = 0.75$ we find

$$\alpha = \frac{1}{c}\log 3 = \frac{1.099}{c} \text{ or } c = \frac{1.099}{\alpha}$$

so that c which is the difference in the natural logarithms of the two doses

which affect 75 and 25% of the population, respectively, is inversely proportional to α.

In the experimental case x_1 and x_2 are known and P_1 and P_2 are observed; from them α and γ may be computed. In many cases α is not desired, but only γ = L.D.50; we may then write

$$\gamma = \frac{x_1 + x_2}{2} + \frac{c}{2}\frac{\log (Q_1/P_1 + \log (Q_2/P_2)}{\log (Q_1/P_1) - \log (Q_2/P_2)}, \; c = x_2 - x_1. \quad (3)$$

The standard deviation of γ may be obtained by the usual method of differentiation.

$$\delta\gamma = \frac{c}{2}\delta\frac{\log (Q_1/P_1) + \log (Q_2/P_2)}{\log (Q_1/P_1) - \log (Q_2/P_2)} = c\frac{\frac{\delta P_1}{P_1Q_1}\log\frac{Q_2}{P_2} - \frac{\delta P_2}{P_2Q_2}\log\frac{Q_1}{P_1}}{[\log (Q_1P_1) - \log (Q_2/P_2)]^2}$$

As P_1 and P_2 are subject to sampling variations of PQ/n, when n is the number of animals in each of the samples (ordinarily the same) to which the two doses are given, and as those sampling variations are independent (non-correlated), we may square, neglecting the product $\delta P_1\, \delta P_2$ and substitute for $(\delta P)^2$ the value PQ/n. Then[2]

$$\sigma_\gamma^2 = \frac{c^2}{n}\frac{\frac{1}{P_1Q_1}\left(\log\frac{Q_2}{P_2}\right)^2 + \frac{1}{P_2Q_2}\left(\log\frac{Q_1}{P_1}\right)^2}{[\log (Q_1/P_1) - \log (Q_2/P_2)]^4} \quad (4)$$

Two biologicals which are standardized at the L.D.50 points will not stay standardized for other dosages unless the two values of α are the same. Thus if $\alpha_1 > \alpha_2$, the first will be relatively stronger than the second above the L.D.50 point, and relatively weaker below it. The standard deviation of α is therefore for the purpose of determining whether the values of α are significantly different. By the process of differentiation,

$$\sigma_\alpha^2 = \frac{1}{4nc^2}\left[\frac{1}{P_1Q_1} + \frac{1}{P_2Q_2}\right]. \quad (5)$$

If there be two values of α each determined by two doses upon n animals

$$\sigma_{(\alpha - \alpha')}^2 = \frac{1}{4nc^2}\left[\frac{1}{P_1Q_1} + \frac{1}{P_2Q_2} + \frac{1}{P_1'Q_1'} + \frac{1}{P_2'Q_2'}\right].$$

The standard deviation of the difference $\gamma - \gamma'$ is

$$\sigma_{(\gamma - \gamma')}^2 = \frac{1}{4n}\left\{\frac{1}{\alpha^2}\frac{\frac{1}{P_1Q_1}\left(\log\frac{Q_2}{P_2}\right)^2 + \frac{1}{P_2Q_2}\left(\log\frac{Q_1}{P_1}\right)^2}{[\log (Q_1/P_1) - \log (Q_2/P_2)]^2} + \right.$$

$$\left. \frac{1}{\alpha'^2}\frac{\frac{1}{P_1'Q_1'}\left(\log\frac{Q_2'}{P_2'}\right)^2 + \frac{1}{P_2'Q_2'}\left(\log\frac{P_1'}{Q_1'}\right)^2}{[\log (Q_1'/P_1') - \log (Q_2'/P_2')]^2}\right\}.$$

If the values of α and α' are different, the result is of less value than if they are the same, because standardization at the L.D.50 points does not ensure standardization at other points.[3]

In this analysis the standard errors of sampling have been obtained as usual by the process of differentiation as is the case with such formulas as $\sigma \log x = \sigma_x/x$. It may, however, be observed that if x may be 0 with a finite probability, $\log x$ must have an infinite value with a finite probability and its standard deviation, strictly considered, must be infinite. Thus such approximations are justifiable only if the probabilities of infinite values are so small that we are willing to disregard them altogether. It is very difficult to discuss this matter in general but an illustration is extremely germane to an understanding of what happens when the numbers n are not very large. Suppose we have two samples of $n = 10$. Then the values of γ and of α and of their standard deviations may be computed for any values of $s_1 = 10P_1$ and $s_2 = 10P_2$ that may be observed on giving the two doses to the two samples. It will be observed from table 1, as is clear from formulas (2)–(5), that many entries in the table are valueless so far as concerns bio-assay. For example, if $s_1 = 0$, $P_1 = 0$ and s_2 is anything other than 10 or 0, formula (3) gives "γ" = 1.0, which means that γ = L.D.50 = x_2, although except when $s_2 = 5$ there is no evidence that γ should be equal to x_2 and the standard error $\sigma_{\text{"}\gamma\text{"}} = \infty$ in these cases only emphasizes this fact. Again, the values of "γ" and "α" when in the diagonal for which $s_1 = s_2$ are useless. Moreover, we are generally dealing with a biological for which we know on *a priori* grounds that negative values of "α" are without meaning. Nevertheless all these embarrassing cases may arise with finite probability from sampling fluctuations no matter what the true values of P_1 and P_2 in the infinite population may be.[4]

In any particular problem of determination of L.D. 50 we should probably know that for the biologicals used $P_2 > P_1$ in the universe and would not be interested in the useless values computed for $s_1 = 0$, $s_2 = 10$ or $s_1 \geqq s_2$. While there would be definite values of "γ" and of "α" obtainable from this universe if P_1 and P_2 were known, we should actually obtain in any one run some one of the values entered in the table, and in the general case it is not necessary that the true universe values appear in the table. Moreover, as the formulas (2) and (3) when $P_1 = s_1/n$ and $P_2 = s_2/n$ are not linear in s_1 and s_2 it cannot be expected that the average of the values obtained when the experiment is run a large number of times are the values in the universe.[5] In like manner the values of $\sigma^2_{\text{"}\gamma\text{"}}$ and $\sigma^2_{\text{"}\alpha\text{"}}$ which we should have to use would be those entered in the table corresponding to the acceptable observed values of s_1 and s_2 and would be very variable, and moreover if the experiment were run many times the scatter of "γ" and "α" computed from the different values of "γ" and "α" that were

TABLE 1

VALUES OF "γ" $= \left[\gamma - \frac{x_1 + x_2}{2}\right]\frac{2}{c}$ AND "α" $= 2c\alpha$, THE REMAINDER TO BE FILLED IN BY SYMMETRY

		$P_2 = 1.0$ $s_2 = 10$	$P_2 = 0.9$ $s_2 = 9$	$P_2 = 0.8$ $s_2 = 8$	$P_2 = 0.7$ $s_2 = 7$	$P_2 = 0.6$ $s_2 = 6$	$P_2 = 0.5$ $s_2 = 5$	$P_2 = 0.4$ $s_2 = 4$
$P_1 = 0.0$	"γ"	?? ± ??	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
$s_1 = 0$	"α"	∞ ± ∞	∞ ± ∞	∞ ± ∞	∞ ± ∞	∞ ± ∞	∞ ± ∞	∞ ± ∞
$P_1 = 0.1$	"γ"	−1.00 ± 0.00	0 ± 0.34	0.23 ± 0.35	0.44 ± 0.38	0.69 ± 0.44	1.00 ± 0.58	1.45 ± 0.92
$s_1 = 1$	"α"	∞ ± ∞	4.39 ± 1.49	3.58 ± 1.32	3.04 ± 1.26	2.60 ± 1.24	2.20 ± 1.23	1.79 ± 1.24
$P_1 = 0.2$	"γ"	−1.00 ± 0.00	−0.23 ± 0.35	0 ± 0.40	0.24 ± 0.47	0.55 ± 0.59	1.00 ± 0.91	1.83 ± 1.98
$s_1 = 2$	"α"	∞ ± ∞	3.58 ± 1.32	2.77 ± 1.12	2.23 ± 1.05	1.79 ± 1.02	1.39 ± 1.01	0.98 ± 1.02
$P_1 = 0.3$	"γ"	−1.00 ± 0.00	−0.44 ± 0.38	−0.24 ± 0.47	0 ± 0.58	0.35 ± 0.78	1.00 ± 1.49	2.84 ± 6.29
$s_1 = 3$	"α"	∞ ± ∞	3.04 ± 1.26	2.23 ± 1.05	1.70 ± 0.98	1.25 ± 0.94	0.85 ± 0.93	0.44 ± 0.94
$P_1 = 0.4$	"γ"	−1.00 ± 0.00	−0.69 ± 0.44	−0.55 ± 0.59	−0.35 ± 0.78	0 ± 1.12	1.00 ± 3.12	∞ ± ∞
$s_1 = 4$	"α"	∞ ± ∞	2.60 ± 1.24	1.79 ± 1.02	1.25 ± 0.94	0.81 ± 0.91	0.40 ± 0.90	0 ± 0.91
$P_1 = 0.5$	"γ"	−1.00 ± 0.00	−1.00 ± 0.58	−1.00 ± 0.91	−1.00 ± 1.49	−1.00 ± 3.12	?? ± ??	−1.00 ± 3.12
$s_1 = 5$	"α"	∞ ± ∞	2.20 ± 1.23	1.39 ± 1.01	0.85 ± 0.93	0.41 ± 0.90	0 ± 0.89	−0.41 ± 0.90
$P_1 = 0.6$	"γ"	−1.00 ± 0.00	−1.45 ± 0.92	−1.83 ± 1.98	−2.84 ± 6.29	− ∞ ± ∞	1.00 ± 3.12	0 ± 1.12
$s_1 = 6$	"α"	∞ ± ∞	1.79 ± 1.24	0.98 ± 1.02	0.44 ± 0.94	0 ± 0.91	−0.41 ± 0.90	−0.81 ± 0.91
$P_1 = 0.7$	"γ"	−1.00 ± 0.00	−2.26 ± 1.93	−4.14 ± 8.04	− ∞ ± ∞	2.84 ± 6.29	1.00 ± 1.49	0.35 ± 0.78
$s_1 = 7$	"α"	∞ ± ∞	1.35 ± 1.26	0.54 ± 1.05	0 ± 0.98	−0.44 ± 0.95	−0.85 ± 0.94	−1.25 ± 0.94
$P_1 = 0.8$	"γ"	−1.00 ± 0.00	−4.42 ± 6.90	− ∞ ± ∞	4.14 ± 8.04	1.83 ± 1.98	1.00 ± 0.91	0.55 ± 0.59
$s_1 = 8$	"α"	∞ ± ∞	0.81 ± 1.32	0 ± 1.12	−0.54 ± 1.05	−0.98 ± 1.02	−1.39 ± 1.01	−1.79 ± 1.02
$P_1 = 0.9$	"γ"	−1.00 ± 0.00	− ∞ ± ∞	4.42 ± 6.90	2.26 ± 1.93	1.45 ± 0.93	1.00 ± 0.58	0.69 ± 0.44
$s_1 = 9$	"α"	∞ ± ∞	0 ± 1.49	−0.81 ± 1.32	−1.35 ± 1.26	−1.79 ± 1.24	−2.20 ± 1.23	−2.60 ± 1.24
$P_1 = 1.0$	"γ"	?? ± ??	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
$s_1 = 10$	"α"	?? ± ??	− ∞ ± ∞	− ∞ ± ∞	− ∞ ± ∞	− ∞ ± ∞	− ∞ ± ∞	− ∞ ± ∞

figured from the observations would not in general be the same as, and might be very different from, the values proper to the universe.[6]

From such considerations it is clear that values of n considerably larger than 10 are necessary to establish that degree of stability in the results figured from the observations which is desirable in any bio-assay. For all values of n the values of "γ" and "α" are as given in the table when the margins are read for P_1 and P_2 (rather than for s_1 and s_2) but the standard deviations would have to be reduced by multiplying by $\sqrt{10/n}$. Furthermore, when n is not 10 the observed values of P may differ from the values entered in the margins and it would be necessary to interpolate by double entry[7] in the table in order to get the values of "γ" and "α." The values of "γ" and "α" and of their standard errors are independent of the logarithmic base, whether e or 10, and the values of γ and α may be obtained in either type by the formulas

$$\gamma = \frac{x_1 + x_2}{2} + \frac{c}{2}\text{“}\gamma\text{”}, \quad \alpha = \frac{\text{“}\alpha\text{”}}{2c}$$

$$\sigma_\gamma = \frac{c}{2}\sigma_{\text{“}\gamma\text{”}}\sqrt{10/n}, \qquad \sigma_\alpha = \frac{\sigma_{\text{“}\alpha\text{”}}}{2c},$$

where x_1, x_2, $c = x_2 - x_1$ are expressed in logarithms to any base. If one wishes to return finally to the actual dosage, D, one has merely to write L.D.50 $= e^\gamma$ or L.D.50 $= 10^\gamma$ according to the base used.

[1] Gaddum, J. H., *Med. Res. Council, Spec. Rep. Series*, No. 183 (1933). Bliss, C. I., *Ann. Applied Biol.*, **22**, 134–167 (1935). Reed, L. J., and Muench, H., *Amer. Jour. Hygiene*, **27**, 493–497 (1938). There is a review of the subject by J. O. Irwin, *Suppl. Jour. Roy. Statistical Soc.* (London), **4**, 1–60 (1937).

[2] Thus, in particular, if we have $P_1 = 0.30$ for a given dose D_1 and $P_2 = 0.75$ for a dose D_2 twice as large, the value of c is log 2 = 0.693, $P_1Q_1 = 0.21$, $P_2Q_2 = 0.1875$, $Q_1/P_1 = {}^7/_3$, $Q_2/P_2 = {}^1/_3$, log $({}^7/_3) = 0.847$, log $({}^1/_3) = -1.099$ and

$$\sigma_\gamma^2 = \frac{(0.693)^2}{n}\,\frac{\frac{1}{0.21}(1.099)^2 + \frac{1}{0.1875}(0.847)^2}{[0.847 + 1.099]^4} = \frac{0.321}{n}$$

$$\gamma = \frac{x_1 + x_2}{2} - 0.0446 \pm \sqrt{\frac{0.321}{n}}.$$

The term ${}^1/_2(x_1 + x_2)$ is the mean logarithmic dose and the 50% end-point is short of it by the amount 0.0446. If n were 20, the standard error would be 0.126; so that we should expect in two-thirds of the runs the value of L.D.50 would lie between 0.081 above and 0.171 below the mean logarithmic dose.

[3] Suppose we have dilutions 1 with $P_2 = 0.75$ and ${}^1/_2$ with $P_1 = 0.30$ of one biological and dilutions ${}^1/_2$ with $P_2' = 0.75$ and ${}^1/_4$ with $P_1' = 0.40$ of a second. We take $x_1 = 0$, $x_2 = 0.693$, $x_2' = 0$, $x_1' = -0.693$. The value of γ is ${}^1/_2(0.693) - 0.0446$ as above with $\sigma_\gamma^2 = 0.321/n$. The value of γ' is $-{}^1/_2(0.693) - 0.159$. The difference between the values is $0.693 - 0.045 + 0.159 = 0.807$. Thus at the L.D.50 points the ratio of

strength is the antilogarithm of 0.807 base e, or 2.24. The values of α are $\alpha = 1.40$ and $\alpha' = 1.09$. The standard errors, if $n = 20$, are $\sigma^2_\alpha = 0.263$, $\sigma^2_{\alpha'} = 0.247$; that of the difference being $\sigma^2 = 0.510$ or $\sigma = 0.71$ which is considerably larger than the difference $\alpha - \alpha' = 0.31$ and indicates that, so far as the test goes, α and α' may be taken as the same. (If we were to take the "best" value for α on the assumption that they were really the same we should then weight the two values according to the reciprocal of the squares of their standard errors, but these are so nearly alike that we could well take the simple mean 1.24.) The value of $\sigma^2{}_{\gamma-\gamma'}$ for $n = 20$ is $0.321/n + 0.554/n = 0.875/n = 0.0438$; hence the difference may be written 0.807 ± 0.209 and is distinctly significant. The relative intensity 2.24 may be given a factor or quotient of the antilogarithm (base e) which is 1.23 or its reciprocal 0.81 or about 21% or 2.24 ± 0.47.

[4] If it be assumed that $P_1 = 0.20$ and $P_2 = 0.80$ in the universe the table of probabilities is (in part, the remainder to be filled in by symmetry):

	$s_2 = 10$	$s_2 = 9$	$s_2 = 8$	$s_2 = 7$	$s_2 = 6$	$s_2 = 5$	$s_2 = 4$
$s_1 = 0$	0.0115	0.0288	0.0324	0.0216	0.0095	0.0028	0.0006
$s_1 = 1$	0.0288	0.0721	0.0811	0.0540	0.0236	0.0071	0.0015
$s_1 = 2$	0.0324	0.0811	0.0912	0.0608	0.0266	0.0080	0.0017
$s_1 = 3$	0.0216	0.0540	0.0608	0.0405	0.0177	0.0053	0.0011
$s_1 = 4$	0.0095	0.0236	0.0266	0.0177	0.0078	0.0023	0.0005
$s_1 = 5$	0.0028	0.0071	0.0080	0.0053	0.0023	0.0007	0.0002
$s_1 = 6$	0.0006	0.0015	0.0017	0.0011	0.0005	0.0002	0.0000
$s_1 = 7$	0.0001	0.0002	0.0002	0.0002	0.0001	0.0000	0.0000

$s_1 = 8$, $s_1 = 9$, $s_1 = 10$ give for all s_2's the probability 0.0000 to four figures.

In this case the troublesome margins for $s_1 = 0$ and for $s_2 = 10$ have a total probability of 0.2033, the troublesome diagonal has an additional probability of 0.0020 and the total probability for $s_1 > s_2$ is 0.0005. This makes a grand total of 0.2058. Had we assumed the $P_1 = 0.40$ and $P_2 = 0.60$ we should have had a smaller probability (0.0120) in the margins for $s_1 = 0$ and $s_2 = 10$, but a larger value (0.2445) for the diagonal $s_1 = s_2$ and below, making an even larger probability of 0.2565 for the cells which are meaningless for the problem of assay.

[5] To find the universe values of γ and α from a large number of runs one would have to add the different s_1's , the different s_2's and the different n's to obtain the probabilities P_1 and P_2 to be used in the formula.

[6] Thus, if, for the universe, $P_1 = 0.20$, $P_2 = 0.80$, the value of $\sigma^2{}_{\text{"}\gamma\text{"}}$ would be 0.162 for $n = 10$ by formula (4), but the different values of "γ" from the experiments would have the probabilities given in the table,[4] and the actual value of $\sigma^2{}_{\text{"}\gamma\text{"}}$ observed in the long run would be 0.215 if we reject the experiments which have unusable constellations (s_1, s_2); and the value of $\sigma^2{}_{\text{"}\gamma\text{"}}$ from the observed distribution of usable values would be 0.86 instead of the value 1.25 given by formula (5).

[7] If $P_1' = P_1 + \theta_1 \Delta P_1$ and $P_2' = P_2 + \theta_2 \Delta P_2$, the interpolation formula is

$$f(P_1', P_2') = f(P_1, P_2) + \theta_1[f(P_1 + \Delta P_1, P_2) - f(P_1, P_2)] + \theta_2[f(P_1, P_2 + \Delta P_2) - f(P_1, P_2)].$$

For a table with such large differences as table 1, the interpolation could not be expected to give a very accurate determination of $f(P_1', P_2')$ but might be adequate.